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(54) Title: REFOLDING AND PURIFICATION OF INSULIN-LIKE GROWTH FACTOR I

(57) Abstract

The present invention resides in the refolding of recombinantly produced IGF-I expressed in prokaryotic cells, particularly bacteria, to render biologically active IGF-I, and to methods of isolating correctly refolded IGF-I from incorrectly refolded IGF-I. The present invention also resides in pharmaceutical compositions containing IGF-I and in methods of treating a patient having an IGF associated condition. The present invention also relates to the conversion of met-IGF-I to IGF-I.

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REFOLDING AND PURIFICATION OF INSULIN-LIKE GROWTH FACTOR I

Field of the Invention

This invention relates to the field of biotechnology processing, more particularly refolding and purification of polypeptides, and even more particularly to the refolding and purification of insulin-like growth factor I.

Background of the Invention and Information Disclosure Statement

The insulin gene family, comprised of insulin, relaxin, insulin-like growth factors I and II, and possibly the beta subunit of 7S nerve growth factor, represents a group of structurally related polypeptides whose biological functions have diverged as reported in Dull, et al., Nature 310:777-781 (1984).

Insulin-like growth factor I (IGF-I), also known as somatomedin C, is a protein of approximately 7.8 kilodaltons. IGF-I binds to IGF receptors. Structural similarity between IGF-I and IGF-II permits both to bind to IGF receptors. Two IGF receptors are known to exist. IGF-I and IGF-II bind to the IGF type I receptor, while insulin binds with less affinity to this receptor. The type I receptor preferentially binds IGF-I and is believed to transduce the mitogenic effects of IGF-I and IGF-II. IGF-II binds to the type I receptor with a 10-fold lower affinity than IGF-I. The second or type II IGF receptor preferentially binds IGF-II. Receptor binding is believed to be necessary for the biological activities of IGF-I and IGF-II.

In addition to specific cell surface receptors, there exist at least six distinct IGF binding proteins (IGFBP-1 through IGFBP-6)

that circulate throughout the body. These proteins bind IGF-I and IGF-II. The binding of IGF-I and IGF-II to binding proteins reduces the action of these IGFs on cells by inhibiting IGF binding to cell surface receptors. Oh et al., Endocrinol. 132:1337-1344, 1993, reports IGF-I and IGF-II are essentially equipotent in their affinities for IGFBP-1. When IGF is bound to binding proteins, it is unable to bind to the IGF receptors and is therefore, no longer active in the body.

IGF-I is mitogenic for a large number of cell types, including fibroblasts, keratinocytes, endothelial cells and osteoblasts (bone-forming cells). IGF-I also stimulates differentiation of many cell types, e.g., synthesis and secretion of collagens by osteoblasts. IGF-I exerts its mitogenic and cell differentiating effects by binding to the specific IGF cell surface receptors. IGF-I also has been shown to inhibit protein catabolism in vivo, to stimulate glucose uptake by cells and to promote survival of isolated neurons in culture. These properties have led to IGF-I being tested as a therapeutic agent for a variety of disease indications as reported in Froesch et al., Trends in Endocrinology and Metabolism, 254-260 (May/June 1990) and Cotterill, Clinical Endocrinology, 37:11-16 (1992).

The role of IGF-I as a useful therapeutic agent for several other disease conditions has also been suggested. For example, IGF-I has long been studied for its role in the growth of various tissues. As reported in Laron et al., Clinical Endocrinology, 35: 145-150 (1991), a marked rise in serum type III procollagen, a

marker of bone formation, occurred after one week of administration of recombinantly produced IGF-I to patients with dwarfism otherwise non-responsive to growth hormone. The effects of the infusion of IGF-I in a child with Laron Dwarfism were described in Walker et al., The New England Journal of Medicine, 324(21):1483-1488 (1991). Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with IGF-I or a truncated form of IGF-I having a deletion of the first three amino acids ordinarily found in IGF-I (referred to as "(des1-3)IGF-I") were demonstrated by Tomas et al., as reported in Biochem. J. 276:547-554 (1991). Growth restoration in insulin-deficient diabetic rats by administration of recombinantly produced human IGF-I was reported in Scheiwiller et al., Nature, 323:169 (1986). IGF-I and (des1-3)IGF-I enhanced growth in rats after gut resection, as reported in Lemmey et al., Am. J. Physiol. 260 (Endocrinol. Metab. 23) E213-E219 (1991). A combination of platelet-derived growth factor and insulin-like growth factors, including IGF-I, enhanced periodontal regeneration in beagle dogs as reported by Lynch et al., J. Clin. Periodontal 16:545-548 (1989). The synergistic effects of platelet-derived growth factor and IGF-I in wound healing were reported in Lynch et al., Proc. Natl. Acad. Sci. 84:7696-7700 (1987). The effects of IGF-I and growth hormone on longitudinal bone growth in vitro were set forth in Scheven and Hamilton, Acta Endocrinologica (Copenhagen) 124:602-607 (1991). In vivo actions of IGF-I on bone formation and resorption in rats were shown in Spencer et al., Bone 12:21-26

(1991). The use of IGF-I and IGF-II for enhancing the survival of non-mitotic, cholinergic neuronal cells in a mammal was described in U.S. Patent 5,093,317 to Lewis et al. In addition, PCT Application Publication No. WO 92/11865 published on July 23, 1992, describes the use of IGF-I for the treatment of cardiac disorders.

Although it is possible to purify IGF-I from human plasma, it is not commercially feasible to do so because of the significant costs involved. Furthermore, proteins purified from human plasma may be contaminated by pathogenic organisms such as viruses including the hepatitis viruses and the AIDS virus. An alternative method for producing large quantities of IGF-I cheaply is to produce it by recombinant DNA methods. With these methods, DNA sequences encoding IGF-I are cloned into a prokaryotic expression vector, for example, pT3XI-2 (described in WO 91/08285), that is capable of directing high level expression of the recombinant proteins in bacteria, particularly Escherichia coli (E. coli). For example, European Patent Application Publication No. 0130166 describes expression of IGF-I in E. coli. This reference does not teach purification nor how to render the protein biologically active.

As an alternative means for obtaining biologically active recombinant IGF-I from bacteria, several groups have constructed gene fusions in which DNA sequences encoding IGF-I are fused to other proteins, protective peptides, or a series of charged amino acid residues. For example, in an attempt to obtain biologically active IGF-I from E. coli, DNA sequences encoding IGF-I were fused

to those encoding a hydrophobic "signal sequence" from lamB or ompF, which directs secretion of the fusion protein into the periplasmic space of E. coli. Endogenous, membrane bound proteases cleave the signal sequence from the mature IGF-I protein. See European Patent Application Publication No. 0288451.

European Patent Application Publication No. 0155655 describes synthesis, bacterial expression and purification of IGF-I fused to other proteins. No data are presented in this reference which demonstrated the activity of this molecule. Similarly, European Patent Application Publication No. 0128733 describes bacterial production of IGF-I fused to other proteins. The fusion protein so produced was cleaved with proteases to release IGF-I. Again, no data was presented in this reference demonstrating the activity of this protein. Fusion proteins yielding five incorrectly folded biologically inactive forms of IGF-I were described in S. Hober et al., Biochemistry 31:1749-1756 (1992). European Patent Application Publication No. 0286345 describes production of human IGF-I in bacteria using a vector in which expression was controlled by a lambda phage promotor and a temperature sensitive repressor protein. The biological activity of the material produced was not demonstrated. Not taught was how to purify the protein, nor whether the N-terminal methionine was cleaved from the protein. IGF-I not having the N-terminal methionine cleaved is referred to as met-IGF-I. PCT Application Publication No. WO91/02807 describes synthesis, expression in bacteria, and refolding of IGF-I fused to charged amino acids at the N-terminus of the protein. The charged

amino acids were added to facilitate refolding of IGF-I; refolding of IGF-I was found to be less than optimal without the charged amino acids. The protein of this reference was refolded and subsequently treated with proteases to remove the extra charged amino acids. The charged amino acids were chosen so that they would be recognized as cleavage sites by diaminopeptidase, beef spleen Cathepsin C.

One of the problems encountered in expressing eukaryotic proteins, particularly those that contain disulfide bonds, in bacteria is that the recombinant proteins are synthesized in an inactive form by the bacteria. Typically, cysteine residues in recombinant proteins expressed in bacteria are improperly paired with one another. The improperly disulfide-bonded proteins tend to be insoluble. This problem has been encountered in previous attempts to express active IGF-I in bacteria. See PCT Patent Application Publication No. WO 89/03423. IGF-I contains six cysteine residues. All six cysteine residues participate in forming disulfide bonds. The cysteine residues must be correctly paired in order for the protein to assume its proper conformation and to exhibit full biological activity. As noted above, attempts at converting the inactive recombinant IGF-I protein into active, properly folded protein by following reactivation and refolding protocols have not been successful due to improper disulfide pairing and low yields. Several groups have set forth various strategies for refolding, renaturing, or reactivating proteins. These include, for example, T. Kohno, et al., Methods in Enzymol.,

185:187-195 (1990). U.S. Patent 4,620,948 reports the refolding of proteins from inclusion bodies of reduced material. U.S. Patent 4,620,948 is deficient in describing purification steps or analytical methods used to isolate and characterize correctly refolded protein. Neither of these references describes refolding of IGF-I to form a biologically active protein. The references also do not describe purification techniques for isolating correctly folded IGF-I from incorrectly folded IGF-I.

Summary of the Invention

In order to overcome the problems discussed above, the present inventors have developed a process for producing and purifying large amounts of biologically active recombinant IGF-I, expressed in prokaryotic cells, particularly in bacteria, and more particularly, in E. coli. The present invention resides in the refolding of recombinantly produced IGF-I expressed in bacteria, to render biologically active IGF-I. The method of refolding of the present invention does not require the construction of fusion proteins or the use of charged amino acids attached to the N-terminus of the protein to produce biologically active IGF-I.

The present invention provides methods whereby inactive met-IGF-I expressed in bacteria can be refolded into its proper conformation. The proteins produced by the instant invention are indicated as being correctly refolded by evidence of their biological activity when compared to a commercially available standard.

The present invention also provides a method for purifying

correctly refolded IGF-I from incorrectly refolded IGF-I.

The present invention also provides a method for converting met-IGF-I to IGF-I.

Also provided in the present invention are pharmaceutical compositions comprising IGF-I and methods of using the IGF-I to treat a patient having or potentially having an IGF associated condition.

Detailed Description of the Invention

Often the lack of biological activity in proteins expressed in microorganisms is related to improper formation of intramolecular disulfide bonds. In the present invention, recombinant IGF-I produced in E. coli may be refolded to attain the correct configuration of intramolecular disulfide bonds and, therefore, exhibit full biological activity.

Terms used throughout this specification are defined as follows:

The term "acceptable pharmaceutical carrier" refers to a physiologically-compatible, aqueous or non-aqueous solvent.

The term "IGF-I" refers to a protein having the same amino acid sequence as naturally occurring IGF-I, or a protein having the same amino acid sequence as naturally occurring IGF-I with the addition of an N-terminal methionine, unless otherwise specified.

The term "IGF associated condition" refers to an existing or potential adverse physiological condition which results from an over-production or underproduction of IGF, IGF binding protein or IGF receptor, inappropriate or inadequate binding of IGF to binding

proteins or receptors and any disease in which IGF administration alleviates disease symptoms. An IGF associated condition also refers to a condition in which administration of IGF to a normal patient has a desired effect.

The term "patient" refers to any animal, including humans, in need of treatment for an IGF associated condition.

The term "denaturing agent, or denaturant" refers to any material which will cause a change in the conformation of a protein that results in a loss of biological activity. Acceptable denaturing agents include, but are not limited to, guanidine and urea.

The term "oxidizing agent" refers to any material which is capable of removing an electron from the compound being oxidized. Acceptable oxidizing agents include, but are not limited to, oxidizing agents which are capable of aiding in the formation of mixed disulfide bonds, for example, oxidized glutathione and cystine.

The term "reducing agent" refers to any material which is capable of adding an electron to a compound. Acceptable reducing agents include any reducing agent capable of the disruption of the molecular disulfide bonds. Acceptable reducing agents include, but are not limited to, dithiothreitol (DTT), 2-mercaptoethanol, and dithioerythritol.

The term "thiol-containing reducing reagent" refers to a reducing agent which contains a sulfhydryl group. Examples include, but are not limited to, dithiothreitol (DTT), 2-

mercaptoethanol, dithioerythritol, cysteine, cystamine, and reducing agents containing added disulfide containing compounds, such as sodium borohydride or any of the Group VIA hydrides containing added cystine, oxidized glutathione or any cysteine-containing dipeptide.

The term "biologically active" refers to the ability to stimulate proliferation of UMR106 rat ostesarcoma cell line, as described in Example 6. The biological activity of a correctly refolded protein stimulates proliferation of the UMR106 cell line at an ED_{50} of about 1 - 30 ng/ml, preferably about 2 - 10 ng/ml and more preferably at about 7 - 8 ng/ml. Incorrectly refolded IGF-I stimulates proliferation of UMR106 rat ostesarcoma cell line with an ED_{50} greater than 30 ng/ml, which for purposes of the present invention is considered "biologically inactive".

The term "ED_{50}" refers to the concentration which causes one-half maximal ^3H incorporation into the DNA of cells.

The recombinant proteins of the present invention were refolded, purified and subsequently treated with an aminopeptidase to remove the extra N-terminal methionine. Aminopeptidases useful for this purpose include, but are not limited to, diaminopeptidase, from beef spleen Cathepsin C, and aminopeptidase from Aeromonas proteolytica.

The examples below set forth the procedures used to construct the IGF-I gene, which was done by forming a gene fusion with a secretory leader sequence for E. coli. From this, a second construct was formed to express met-IGF-I without the secretory

leader sequence. The plasmid thus created was used to transform E. coli to express the met-IGF-I. The yield of met-IGF-I expressed exceeds 10% of total cell protein. The protein thus expressed was purified after disrupting the E. coli cells. The insoluble biologically inactive met-IGF-I was rendered soluble and biologically active by use of a refolding procedure. Properly refolded IGF-I was isolated from improperly refolded IGF-I by use of several column chromatography procedures.

The instant invention resides in the refolding and purification of the resultant recombinant protein to render biologically active IGF-I. In the present invention, recombinant IGF-I may be refolded by using the following steps:

(1) Any intramolecular or intermolecular disulfide bonds and/or any noncovalent interactions which have occurred involving the mature IGF-I produced in a microorganism are first disrupted. In order to do this, the protein is exposed to sufficient denaturant (for example, guanidine hydrochloride or urea) and sufficient reducing agent (for example, beta-mercaptoethanol, dithiothreitol, or cysteine) to denature the protein, disrupt noncovalent interactions, and reduce disulfide bonds.

(2) After the mature IGF-I has been denatured and reduced, the free thiols present in the reduced protein are oxidized by addition of a large excess of disulfide-containing oxidizing agent (for example, oxidized glutathione or cystine). This reaction produces mixed disulfide bonds in which each cysteine residue in the mature IGF-I forms a disulfide bond with the monomeric form of

the oxidizing agent. This step helps to prevent the formation of incorrect intramolecular disulfide bonds in the IGF-I during subsequent processing.

(3) The denaturant and oxidizing agent are then diluted to a defined concentration and a then second reducing agent, also known as a thiol-containing reducing reagent, is added to catalyze disulfide interchange. The objective is to produce an environment in which the denaturant is sufficiently reduced to allow the IGF-I to assume various 3-dimensional configurations and in which the oxidization/reduction potential is adjusted to allow the formation and breaking of disulfide bonds. It is believed that the proper 3-dimensional structure and disulfide bonding pattern of the mature IGF-I is energetically more stable than other possible conformations. Therefore, under conditions in which the IGF-I is allowed to assume a variety of 3-dimensional conformations and intramolecular disulfide bond patterns, a significant proportion of the IGF-I will form the correct intramolecular disulfide bonding pattern assuming the correct 3-dimensional structure, and, therefore, become biologically active.

These procedures are mild and should not result in the chemical modification of the IGF-I. If urea is used as a denaturant, any interfering cyanate that may form can be removed by passing the urea solution over an anion exchange column, such as DOWEX 1-X8(BioRad). Cyanate can modify amino groups in the protein (Stark, Methods in Enzymology 11:125 1967).

The optimal concentration and choice of denaturant, oxidizing

agent, thiol-containing reducing reagent and their concentrations in the final refolding solution are determined experimentally by monitoring the proportion of IGF-I properly refolded and biologically active. The objective in the final refolding solution is to provide a controlled environment in which disulfide interchange and conformational changes can occur in the IGF-I until the favored conformation and disulfide bonding pattern is achieved.

In an embodiment of the present invention, the IGF-I is substantially purified from soluble proteins prior to refolding. An alternative embodiment is contemplated whereby the IGF-I is substantially purified from soluble and insoluble proteins prior to refolding. Substantially purified in this context means the solution is substantially free of host cell proteins that interfere with the rate or efficiency of IGF-I refolding.

The correctly refolded IGF-I is separated from the incorrectly refolded IGF-I by means of various column chromatography techniques, including, for example, the techniques described below in Example 4. In one embodiment of the separation method, the first step is dialysis to decrease the amount and concentration of reducing agent and denaturing agent used in the refolding process. The second step utilizes an ion exchange column which separates protein isomers according to charge. Although Example 4 teaches the use of an S-sepharose column for this purpose, those skilled in the art can readily determine other cation exchange columns that could be used for this purpose. This S-sepharose chromatography procedure of Example 4 yielded two major peaks. The peak

corresponding to correctly folded protein was identified by comparison to a commercial standard. The ED_{50} of this peak (Peak B) was 7-8 ng/ml when measured by the UMR106 cell assay described in Example 6. The ED_{50} of the other peak (Peak A) was 30 - 40 ng/ml when measured by the same assay. Peak A was determined to be incorrectly folded protein. The final step for separating correctly folded protein from incorrectly folded protein can be reverse phase HPLC, which is particularly useful for small scale experiments. Alternatively, a hydrophobic interaction column can be used in the final step. The hydrophobic interaction column separates the proteins based on their hydrophobicity. Any hydrophobic interaction chromatography column, such as, for example, Toyopearl Butyl-650S, can be used for this purpose. The isolated, correctly folded protein can then be analyzed using reverse phase HPLC, if desired.

Although IGF-I having an N-terminal methionione (met-IGF-I) exhibits biological activity comparable to IGF-I as demonstrated in the bioassays described below (Example 6), it may be desirable to cleave the N-terminal methionine from the IGF-I. Since naturally occurring IGF-I has no N-terminal methionine, met-IGF-I may give rise to an immune response in some circumstances. For that reason, the present invention also provides a method for converting met-IGF-I to IGF-I. This is accomplished by reacting the met-IGF-I with an aminopeptidase, for example, an aminopeptidase from Aeromonas proteolytica, to cleave the N-terminal methionine. The reaction is stopped by lowering the pH of the solution to below pH

5. This can be accomplished by the addition of any of several acids. Suitable acids for this purpose include, but are not limited to, trifluroacetic acid (TFA), acetic acid, and hydrochloric acid. This reaction can also be stopped by lowering the temperature to below 4°C.

The present invention further provides a pharmaceutical composition containing IGF-I in a pharmaceutically acceptable carrier. One carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one embodiment it is envisioned that the carrier and the IGF-I constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the IGF-I. Such excipients are those substances usually and customarily employed to formulate dosages for administration in either unit dose or multi-dose form.

Once the pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or

requiring reconstitution immediately prior to administration. The storage of such formulations can be at temperatures at least as low as 4°C and preferably at -70°C. Formulations containing IGF-I can also be stored and administered at or near physiological pH. It is presently believed that storage and administration in a formulation at a high pH (i.e. greater than 9) or at a low pH (i.e. less than 2) is undesirable.

The pharmaceutical composition of the present invention can be used to treat a patient having or potentially having an IGF associated condition. Some of these conditions may include, for example, dwarfism, diabetes, cachexia, peripheral neuropathy, renal disease, impaired wound healing, amyotrophic lateral sclerosis (ALS), stroke, periodontal disease and osteoporosis. The pharmaceutical composition of the present invention can also be used to treat a condition in which administration of IGF to a normal patient has a desired effect; for example, using IGF-I to enhance growth of a patient of normal stature.

The manner of administering the formulations containing IGF-I can be via an intraarticular, subcutaneous, intramuscular or intravenous injection or infusion, suppositories, enema, inhaled aerosol, or oral or topical routes. To achieve and maintain the desired effective dose of IGF-I, repeated subcutaneous or intramuscular injections may be administered. Both of these methods are intended to create a preselected concentration range of IGF-I in the patient's blood stream. It is believed that the maintenance of circulating concentrations of IGF-I of less than

0.01 ng per ml of plasma may not be effective, while the prolonged maintenance of circulating levels in excess of 100 μg per ml may be undesirable. The frequency of dosing will depend on pharmacokinetic parameters of the IGF-I in the formulation used.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE 1

IGF-I

A. Construction of the IGF-I gene

The IGF-I gene was assembled in two stages. Initially, the DNA sequence encoding the mature IGF-I protein was joined to DNA sequences encoding the secretory leader sequence of the E. coli OmpA protein ($ompA_L$). This gene fusion was constructed in order to determine whether IGF-I could be efficiently secreted from E. coli. A second construct, in which IGF-I is expressed as an intracellular protein, was created by deleting DNA sequences encoding the OmpA leader sequence and replacing them with appropriate DNA sequences for intracellular expression of IGF-I.

B. Construction of the $OmpA_L$-IGF-I gene fusion

The four synthetic oligonucleotides labeled OmpA1U (SEQ ID NO:1), OmpA2U (SEQ ID NO:2), OmpA1L (SEQ ID NO:3) and OmpA2L (SEQ ID NO:4), were annealed pairwise (1U + 1L and 2U + 2L) and the pairs ligated together. All four of these oligonucleotides were synthesized using DNA synthesizers purchased from Applied Biosystems (Models 391 and 380A). The ligation mixture was then digested with the restriction enzyme HaeIII. The resulting

BamHI/HaeIII restriction fragment coding for a translational start signal and the first 21 amino acids of the ompA signal sequence was purified. This DNA fragment was mixed with BamHI + PstI-digested pUC18 DNA (Boehringer Mannhein Biochemicals, Indianapolis, IN) and the two synthetic oligonucleotides [IGF-I (1-14) U + L] (SEQ ID NO:5 and SEQ ID NO:6) were ligated together. The ligation mixture was transformed into E. coli strain JM109 (New England Biolabs, Beverly, MA) and individual colonies isolated. These plasmids ($OmpA_L$IGF-IpUC18) have a translational start signal followed by DNA sequences encoding the OmpA signal sequence and the first 14 amino acids of IGF-I.

An M13 phage containing DNA sequences encoding amino acids 15 through 70 of IGF-I was created by ligating together the two complementary pairs of oligonucleotides (IGF1U + 1L and IGF2U + 2L) (SEQ ID NO:7 and SEQ ID NO:8) and cloning the DNA fragment into PstI + HindIII-digested M13 mp19 DNA (New England Biolabs, Beverly, MA). Double-stranded DNA was purified from a phage clone and the PstI/HindIII fragment encoding amino acids 15-70 of the IGF-I protein were isolated. This DNA fragment was ligated together with PstI + HindIII-digested plasmid $OmpA_L$IGF-IpUC18 DNA and used to transform E. coli strain JM107 (GIBCO BRL, Gaithersburg, MD). The BamHI/HindIII fragment containing the IGF-I gene fused to the $OmpA_L$ sequence was isolated and cloned into the BamHI + HindIII generated site of plasmid pT3XI-2. The completed plasmid containing the $OmpA_L$-IGF-I gene fusion is called pT3XI-2 $\phi 10_C$(TC3)$ompA_L$IGF-I.

C. Construction of the Methionyl-IGF-I gene

The BamHI/HindIII fragment containing the $OmpA_L$-IGF-I gene described above was purified from plasmid pT3XI-2$\phi10_C$(TC3)$ompA_L$IGF-I and digested with HinfI. The approximate 200 bp HinfI/HindIII DNA fragment was mixed with the annealed, complementary synthetic oligonucleotides (MetIGF1U + 1L) (SEQ ID NO:9 and SEQ ID NO:10) and ligated with BamHI + HindIII-digested plasmid pT3XI2 DNA, and used to transform E. coli JM107. The completed plasmid construct is called $\phi10_C$(TC3)IGF-IpT3XI-2 and contains an extra alanine residue at the beginning of the IGF-I sequence. The BamHI/HindIII fragment containing the mutant IGF-I gene was isolated and ligated into the BamHI + HindIII generated site of plasmid pT5T (described in Nature, Vol. 343, No. 6256, pp. 341-346). The ligation mixture was used to transform E. coli BL21/DE3 (US Patent 4,952,496) and individual colonies isolated. This construct was named $\phi10_C$(TC3)IGF-IpT5T.

The extra alanine codon was removed by in vitro mutagenesis. Plasmid $\phi10_C$(TC3)IGF-IpT3XI-2 was digested with BamHI + HindIII and the ~200 bp DNA fragment containing the mutant IGF-I gene was purified and cloned into the BamHI and HindIII sites of plasmid M13 mp19. In vitro mutagenesis was performed using a Muta-Gene kit (Bio-Rad Laboratories, Richmond, CA). The procedure followed was described in the instructions that accompany the kit. Uracil-containing single-stranded template DNA was prepared following propagation of the phage in E. coli strain CJ236 (supplied with Muta-Gene Kit, Bio-Rad Laboratories, Richmond, CA). The oligonucleotide used for mutagenesis had the sequence: 5' -

GATGATTAAATGGGTCCGGAGACT - 3' (SEQ ID NO:11). The mutagenesis reaction product was transformed into E. coli strain JM109 and individual plaques picked. Double-stranded replicative form phage DNA was isolated, digested with BamHI + HindIII and the ~200 bp fragment containing the IGF-I gene purified. The purified DNA was cloned into the BamHI + HindIII generated site of plasmid pT5T and used to transform E. coli strain BL21/DE3. One bacterial colony with the correct plasmid was named ϕ10(TC3)mutIGF-IpT5T. Several isolates were sequenced, and all were correct.

D. Expression of Met-IGF-I in bacteria

For small-scale experiments, an overnight culture of E. coli strain containing ϕ10(TC3)mutIGF-IpT5T was diluted 1:100 into 800 ml of Luria Broth (10 g/liter tryptone, 5 g/liter yeast extract and 10 g/liter NaCl, pH 7.5) medium containing 15 μg/ml tetracycline and grown at 37° until the optical density at 600 nm was 0.7-0.9. IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma Chemical Company, St. Louis, MO) was added to a final concentration of 1 mM and the culture grown for an additional 2.5-3.0 hours at 37°C. At the end of the induction period, the cells were harvested by centrifugation. The cell pellet was washed once with ice-cold buffer A (50 mM Tris-HCl pH 7.5/ 25 mM NaCl/1 mM DTT) and stored at -70°C or resuspended in buffer A and used immediately.

For large-scale experiments, E. coli strain ϕ10(TC3)mutIGF-IpT5T was grown in a 10 l fermenter at 37°C in complex media (40 g/l NZ amine HD, 2 g/l KH_2PO_4, 1 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l Na_2SO_4, 1 g/l Na_3 citrate $\cdot 2H_2O$, 50 g/l glycerol, 0.1 ml/l Macol 19::GE60, 2 ml/l

trace minerals, 20 mg/l thiamine HCl, and 15 mg/l tetracycline HCl, pH 7). When the optical density at 600 nm of the culture reached approximately 10, IPTG was added to a final concentration of 0.1 mM. Bacteria were grown for an additional 2-8 hours, harvested by centrifugation and the cell pellet stored at -70°C until use.

EXAMPLE 2

Purification of Met-IGF-I

E. coli cells were suspended in Buffer A (50 mM Tris, pH 7.5, 20 mM NaCl and 1 mM DTT), and were disrupted at 1800 psi using a French pressure cell. The suspension was centrifuged at 20,000 x g for 30 minutes, and aliquots of the pellet and the supernatant were analyzed by SDS-PAGE. A major band corresponding to met-IGF-I was present in the pellet, but not the supernatant. The pellet was resuspended in Buffer A (40 ml/10 g cell paste), and re-centrifuged at 20,000 x g for 30 min. This wash procedure was repeated 2 times. The final pellet containing met-IGF-I was resuspended in 6 M guanidine, 50 mM Tris, pH 7.5, 6 mM DTT (25 ml/10 g cell paste) using a ground glass homogenizer, and the suspension was incubated at room temperature for 15 minutes. The undissolved protein was removed by centrifugation at 20,000 x g for 30 minutes. SDS-PAGE analysis of the pellet and supernatant showed that met-IGF-I was present in the supernatant only.

EXAMPLE 3

Refolding of Met-IGF-I

The reduced met-IGF-I from Example 2 was subjected to a three-step refolding protocol.

1) The oxidizing agent, oxidized glutathione (GSSG), was added to the supernatant from Example 2 to a final concentration of 25 mM, and incubated at room temperature for 15 minutes.

2) The solution was then diluted 10 fold gradually with 50 mM Tris, pH 9.7 to a final concentration of 100 - 200 µg/ml. Cysteine was added to a final concentration of 5 mM to aid in disulfide exchange.

3) The solution from step (2) was incubated overnight at 4°C to allow completion of disulfide exchange, and then centrifuged at 20,000 x g for 15 minutes. SDS-PAGE analysis of the pellet and the supernatant showed that the supernatant was composed of relatively homogeneous met-IGF-I.

Aliquots (50 µl) of the supernatant were diluted to 1 ml with 0.05% TFA, injected onto a reverse phase column (RP-4, 1 x 250 mm, SynChrom), and eluted with Buffer B (80% acetonitrile in water, 0.042% TFA) using a linear gradient (increase of 1% Buffer B/min) at a flow rate of 0.1 ml/minute.

Two major peaks were resolved: Peak I at 56.5 minutes, and Peak II at 58.2 minutes. In addition, a minor peak was present at 60 minutes, and a broad peak at 75-79 minutes containing improperly refolded met-IGF-I species. Based on the integration of the HPLC chromatogram, Peak I and Peak II represented approximately 25% and 30% of the crude met-IGF-I protein loaded onto the reverse phase column, respectively. N-terminal sequence analysis of Peak I and Peak II gave the sequence MetGlyProGluThrLeu... (SEQ ID NO:12), which matches the N-terminal amino acid sequence of human IGF-I

except for the extra methionine residue at the N-terminus. Recombinant human met-IGF-I (purchased from Bachem, Torrance, CA) eluted at a retention time identical to Peak II. Therefore, Peak II represents correctly refolded met-IGF-I, as evidenced by retention time identical to the purchased standard as well as biological activity identical to the purchased standard. IGF-I which has not been correctly refolded exhibits reduced or no biological activity. Correctly refolded IGF-I is evidenced by ED_{50} of 10 ng/ml or less when tested on the UMR106 cell line. This assay is described in Example 6.

EXAMPLE 4

Isolation of Correctly Refolded IGF-I

The following is a description of the preparation of IGF-I from 305 g of cell paste. The supernatant from the refolding procedure (6700 ml) was concentrated 10-fold and dialyzed to completion against 20 mM HEPES, pH 7.5. The dialyzed sample was centrifuged 20,000 X g for 15 minutes to remove precipitated proteins, passed through a 0.2 μm filter (Corning, Corning, NY) and loaded onto an S-Sepharose column (5.0 X 40 cm, Pharmacia LKB, Piscataway, NJ) previously equilibrated with the same buffer, at a flow rate of 40 ml/minute. The bound IGF-I was eluted with a 5000 ml linear gradient to 0.5 M NaCl at a flow rate of 40 ml/minute. 25 ml fractions were collected. Two symmetrical peaks were resolved: Peak A eluting at 0.12 M NaCl, and Peak B eluting at 0.15 M NaCl. SDS-PAGE analysis of aliquots of Peaks A and B showed that they contained relatively homogeneous IGF-I (> 90% homogeneous);

however, several high molecular weight E. coli proteins were still present. The S-Sepharose fractions corresponding to Peaks A and B were pooled separately. HPLC analysis (RP-4, 1 x 250 mm) of the S-Sepharose pools showed that Pools A and B were composed of major peaks eluting at 56.5 minutes and 58.2 minutes, respectively, as well as several minor peaks. The major RP-4 peak of the S-Sepharose pool B eluted with the same retention time as commercially purchased recombinant human met-IGF-I (Bachem, Torrance CA).

The S-Sepharose pool B was made to 2 M NaCl, 20 mM HEPES, pH 7.5, and loaded at a flow rate of 30 ml/minute onto a Toyopearl Butyl-650S (Supelco, Bellefonte, PA) hydrophobic interaction column previously equilibrated with 20 mM HEPES, pH 7.5, 2M NaCl. The bound protein was eluted with a 1250 ml linear gradient to 20 mM HEPES, pH 7.5, 20 % ethanol at a flow rate of 40 ml/minute. 25 ml fractions were collected. A major peak eluted at approximately 17.5 % ethanol, as well as a minor peak at 13-15 % ethanol. Aliquots (50μl) of the fractions were diluted to 200μl with Buffer C (0.05% TFA), injected onto a reverse phase column (RP-4, 1 x 250mm, Synchrom), and eluted with 80% acetonitrile, 0.042% TFA (Buffer D) using a linear gradient (increase of 1% Buffer D/minute) at a flow rate of 0.1 ml/minute. The major peak eluting at 17.5% ethanol contained homogeneous, correctly refolded IGF-I. This major peak was determined to contain correctly refolded IGF-I because it eluted with the same retention time as commercially purchased natural human met-IGF-I. (Bachem, Torrance, CA)

Fractions containing this peak were pooled, concentrated to 2 mg/ml, dialyzed against 100 mM HEPES, 44 mM sodium phosphate, pH 6.0, and stored at -70°C.

EXAMPLE 5

Conversion of Met-IGF-I to IGF-I

In order to convert recombinant met-IGF-I to natural human IGF-I, an aminopeptidase, isolated from Aeromonas proteolytica using a modification of a previously described method (Lorand, L., 1976, Meth. Enzymol. 45:530-543), incorporated herein by reference, was used to remove the N-terminal methionine. Recombinant met-IGF-I was incubated in the presence of the purified aminopeptidase in a 100 µl reaction mixture containing 120 µg met-IGF-I, 20 mM Tricine, pH 8.0, and 1 µg aminopeptidase for 30 minutes at 25°C. The reaction was stopped by the addition of 1 ml 0.05% TFA in water. Aliquots of the samples were analyzed on a reverse phase column, and the protein peaks collected and subjected to sequence analysis. met-IGF-I eluted at 58.2 minutes; whereas, the material reacted with the aminopeptidase comigrated with natural human IGF-I (purchased from Bachem, Torrence, CA) at 56 minutes. The following is a summary of the pmoles of each residue recovered at each sequence cycle, normalized for 100 pmoles of starting material:

TABLE 1

Met-IGF-I + 1 μg Aminopeptidase

	Pmoles Recovered				
Cycle	Met	Gly	Pro	Glu	Thr
1	2.32	86.8	2.4	1.78	1.45
2	0.00	23.3	105.6	3.1	0.8
3	0.18	13.8	23.4	128.6	1.4
4	0.00	9.3	4.6	24.1	51.5

Sequence obtained: Gly, Pro, Glu, Thr; approximately 2% of the molecules did not have N-terminal Met cleaved by aminopeptidase.

TABLE 2

Met-IGF-I No Aminopeptidase

	Pmoles Recovered				
Cycle	Met	Gly	Pro	Glu	Thr
1	88.16	11.6	2.15	1.62	0.0
2	4.8	108.4	2.31	4.1	2.0
3	0.5	25.8	80.72	7.6	1.3
4	0.0	15.9	25.2	71.6	0.9

Sequence obtained: Met, Gly, Pro, Glu

These results show that approximately 98% of met-IGF-I was converted to natural IGF-I by the aminopeptidase.

EXAMPLE 6

Biological Activities of Recombinant Met-IGF-I

A. In vitro Activities

The in vitro biological activities of purified recombinant met-IGF-I were tested in cell proliferation assays using mouse 3T3

fibroblasts, and on rat osteosarcoma UMR106 cells. The cell proliferation assay used is set forth below.

1. Effect of Met-IGF-I on Mouse 3T3 Fibroblasts

A crystal violet dye assay was used to measure cell proliferation. Assays were performed in 96 well gelatin-coated plates. Balb/c 3T3 fibroblasts (available from American Type Culture Collection, Rockville, MD, Accession #CCL 163) were plated at 25,000 cells/well in 200 µl of serum-free DMEM (Dulbecco's Modification of Eagle's Medium, Mediatech, Herndon, VA) containing 0.03 M glycerol and 0-1,000 ng/ml met-IGF-I. Cells were incubated for 72 hours at 37°C. At this time, the media was replaced with 150 µl of 0.2% crystal violet, 10% formaldehyde, 10 mM potassium phosphate pH 7.0. After incubation at room temperature for 20 minutes, the wells were washed 3 times with PBS, and the cell-bound dye was released by incubation with 200 µl/well of 50% ethanol/0.1M sodium citrate, pH 4.2. Absorbance at 570 nm was read the next day. The results show that recombinant met-IGF-I stimulates proliferation of 3T3 fibroblast cells in a dose-dependent manner. Maximal proliferation occurred at met-IGF-I concentration of about 30 ng/ml. The ED_{50} was approximately 2 ng/ml.

2. Effect on rat osteosarcoma UMR106 cells

The mitogenic (growth stimulating) activity of the refolded met-IGF-I was measured by the amount of ^{3}H-thymidine incorporated into rat osteosarcoma cells when the met-IGF-I was incubated with these cells under serum free conditions. The rat osteosarcoma cells (the UMR106 cell line; American Type Culture Collection,

Accession No CRL-1661, Rockville, Maryland) were plated at 5-6 X 10^4 cells in 0.5 ml of Ham's F12 (Cat. #10-080-LV, Mediatech) containing 7% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine per well in 48-well tissue culture plates (Costar, Cambridge, MA). After incubating for 72 hours at 37°C when the cells became confluent, the cells were washed twice with PBS and pre-incubated in serum-free Ham's F12 medium containing 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine for 24 hours. After the pre-incubation, 0.5 ml of F12 serum-free medium containing serial dilutions (1.0 - 1,000 ng/ml) of met-IGF-I, were incubated for an additional 20-24 hours. Each well was then pulse labeled with 0.5 uCi of ^{3}H-thymidine (Cat. #NET-027Z, NEN Research Products, Du Pont Co., Boston, MA) for 4 hours, then washed with cold PBS three times and incorporated ^{3}H-thymidine was precipitated with cold 7% trichloroacetic acid (Cat. #0414-01, J.T. Baker Inc., Phillipsburg, NJ). ^{3}H-thymidine was quantitated by liquid scintillation counting. All assays were performed in triplicate. The results show that recombinant met-IGF-I stimulates proliferation of rat UMR106 cells in a dose dependent manner. The ED_{50} of refolded met-IGF-I was 2 - 20 ng/ml.

B. In vivo activities of recombinant Met-IGF-I

IGF-I possesses both growth-promoting and metabolic properties similar to those of insulin (L. Rossetti. Diabetes 40:444-448, 1991). We have demonstrated in rats that met-IGF-I possesses both growth-promoting and metabolic effects and is therefore bioactive.

1. Growth of hypophysectomized rats is promoted by the

subcutaneous injection of Met-IGF-I

Hypophysectomized rats are deficient in both growth hormone (GH) and IGF-I. GH is believed to stimulate growth indirectly by inducing synthesis of IGF-I which then acts directly on tissues to regulate growth. Although the hypophysectomized rat is stunted, growth can be stimulated by the administration of either IGF-I or GH. Subcutaneous infusion of IGF-I purified from natural sources stimulates an increase in body weight and tibial epiphyseal width (Schoenle, E. Nature 296:252-253, 1982).

Male Sprague Dawley rats which were surgically hypophysectomized at 120-130 grams of body weight were obtained from a commercial source (Charles River, Wilmington, MA). The body weights of these rats were monitored for three weeks before the beginning of the experiment in order to verify completeness of the hypophysectomy. Rats gaining more than 2 grams per week were excluded from the study.

The rats were divided into two groups containing four rats in each. One group was injected subcutaneously at the nape of the neck with recombinant met-IGF-I produced as set forth above (80 μg/rat/injection) twice a day at 9:00 a.m. and 8:00 p.m. for nine consecutive days. The other group of four rats was injected with an equal volume of vehicle (0.2 ml). Body weights were measured daily at the time of the morning injection. Twelve hours after the last injection, the rats were killed, and the right and left tibias were removed. The formalin-fixed tibias were split at the proximal end in a sagittal plane and stained with silver nitrate (Greenspan,

F.S., Endocrinology 45:455-463, 1949). The calcified tissue was stained dark brown and the proliferating zone of cartilage appeared as a clearly defined white band. The cartilaginous epiphyseal plate was measured with a stereomicroscope with a calibrated micrometer eyepiece. Approximately ten individual readings were made across the width of the epiphysis. The mean of the combined readings from the right and left tibias was calculated for each rat.

A significant change in body weight occurred over a nine day period in four met-IGF-I-injected rats, but not in four vehicle-injected rats. The first injections were given on day zero. The change in body weight was calculated as the difference between the body weight at each subsequent day of injection and that on day zero. The increase in body weight in the met-IGF-I-treated rats was significantly greater than the change in weight in the vehicle-treated rats on days 3-9. Overall during the nine day period, the met-IGF-I-treated rats gained an average of 8.3 ± 0.5 grams of body weight per rat; whereas, the body weights of the vehicle-treated rats remained stabilized with a change of only 1.0 ± 1.2 grams on average per rat. The difference between the two group is statistically significant ($p<0.05$ using an unpaired t test).

The width of the epiphyseal cartilage of the met-IGF-I-treated rats was greater than that of the vehicle-treated rats. The epiphyseal widths were 0.20 ± 0.01 mm in met-IGF-I-treated rats and 0.14 ± 0.01 in vehicle-treated rats. The difference between the two groups is statistically significant ($p<0.005$ using an unpaired

t test).

2. Hypoglycemia is induced by the intravenous injection of recombinant Met-IGF-I

The intravenous, bolus injection of IGF-I purified from natural sources provokes a rapid fall in blood sugar (Zapf, A.J., Clin. Invest. 77:1768-1775, 1986). In order to determine the effect of intravenously injected met-IGF-I produced in accordance with the present invention on blood glucose levels, hypophysectomized rats weighing between 120 and 140 grams were tranquilized by the subcutaneous administration of diazepam (11 mg/kg). At time zero, blood samples were obtained from the tail vein for determination of the pre-injection blood glucose levels. Intravenous injections of either met-IGF-I at 5, 12.5, and 17.5 μg/rat, or vehicle, were administered through the tail vein. There were three rats per dosage group of met-IGF-I. Four rats were injected with vehicle. Blood samples were obtained at 15 minute intervals during the 120 minute period thereafter. One drop of blood (about 50 μl) was collected by sequential nicks of the rat's tail at a single site superficial to the lateral tail vein. Blood glucose levels were measured immediately on an Ames glucometer (Miles Inc., Elkhart, IN).

Blood glucose levels were not significantly lowered in rats injected with either vehicle or 5 μg met-IGF-I per rat. In fact, these rats were probably undergoing a stress-induced hyperglycemia due to experimental manipulations. However, rats injected with 12.5 and 17.5 μg met-IGF-I experienced a marked drop in blood

glucose levels. Glucose values decreased by 40% of the time zero values.

Although this invention has been described with respect to specific embodiments, it is not intended to be limited thereto and modifications made by those skilled in the art are considered to fall within the spirit and scope of the instant invention.

(1) GENERAL INFORMATION
(i) APPLICANT: SYNERGEN, INC.

(ii) TITLE: Refolding of Insulin-Like Growth Factor I

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.5 inch, 360 Kb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: MS DOS
(D) SOFTWARE: Wordperfect 5.1

(vi) CURRENT APPLICATION DATA: NONE

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(A) APPLICATION NUMBER: 07/858,161
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAT CCG ATC GTG GAG GAT TAA ATG AAA AAG ACA GCT ATC GCG ATC 48

GCA 51

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GCT CCG TGG CAG 48

TGC 51

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAG TGC CAC TGC GAT CGC GAT AGC TGT CTT TTT CAT TTA ATC CTC 48

CAC GAT CG 56

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCA CTG CCA CGG AGC GGC CTG CGC TAC GGT AGC GAA ACC AGC 42

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CC GGT CCG GAG ACT CTG TGC GGC GCA GAA CTG GTT GAC GCT CTG CA 46

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAG CGT CAA CCA GTT CTG CGC ACA GAG TCT CCG GAC CGG 42

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTC GTA TGC GGC GAC CGT GGC TTC TAC TTC AAC AAA CCG ACT GGC TAC 49
GGT TCC AGC TCT CGT GCA CCG CAG ACT GGT ATC 85

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 98 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTC GTC AAC GAT ACC AGT CTG CGG TGC ACG AGA GCT GGA ACC GTA 48
GCC AGT CGG TTT GTT GAA GTA GAA GCC ACG GTC GCC GCA TAC GAA CTG 96
CA 98

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCCGATCG TGGAGGATGA TTAAATGGCC GGTCCGGAG 39

(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGTCTCCGGA CCGGCCATTT AATCATCCTC CACGATCG 38

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATGATTAAA TGGGTCCGGA GACT 24

(13) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Gly Pro Glu Thr Leu

We claim:

1. A method for producing a biologically active recombinant IGF-I, comprising the steps of:

a) obtaining a solution of recombinant IGF-I expressed by a prokaryotic cell;

b) adding a first reducing agent to the solution to form a reduced solution;

c) adding a denaturing agent simultaneously with or after the first reducing agent to form a denatured solution;

d) adding an oxidizing agent to the denatured solution to form an oxidized solution;

e) adding a second reducing agent to the oxidized solution to produce the biologically active recombinant IGF-I.

2. The method of claim 1, wherein the prokaryotic cell is a bacterium.

3. The method of claim 2, wherein the bacterium is E. coli.

4. The method of claim 3, wherein the E. coli contains ϕ10(TC3)mutIGF-IpT5T.

5. The method of claim 1, wherein the oxidizing agent is a disulfide containing compound.

6. The method of claim 5, wherein the disulfide containing

compound is oxidized glutathione or cystine.

7. The method of claim 1, wherein the reducing agent of step (b) is selected from the group consisting of dithiothreitol (DTT), 2-mercaptoethanol, and dithioerythritol.

8. The method of claim 1, wherein the denaturing agent is guanidine or urea.

9. The method of claim 1, wherein the second reducing agent of step (e) is a thiol-containing reducing reagent.

10. The method of claim 9, wherein the thiol-containing reducing reagent is selected from the group consisting of: dithiothreitol (DTT), 2-mercaptoethanol, dithioerythritol, cysteine, cystamine, reduced glutathione, and a reducing agent containing an added disulfide containing compound.

11. The method of claim 1, further comprising isolating properly refolded recombinant IGF-I from improperly refolded recombinant IGF-I after step (e).

12. The method of claim 11, wherein the properly refolded recombinant stimulates proliferation of UMR106 rat osteosarcoma cell line with an ED_{50} of 2 - 10 ng/ml.

13. The method of claim 1, further comprising:

adding an effective amount of an aminopeptidase after step (e) to cleave the N-terminal methionine; and stopping the reaction.

14. The method of claim 13, wherein the reaction is stopped by lowering the pH of the solution to below pH 5.

15. The method of claim 14, wherein the pH of the solution is lowered to below pH 5 by the addition of trifluoroacetic acid.

16. A pharmaceutical composition comprising biologically active IGF-I prepared according to the method of claim 1 in an acceptable pharmaceutical carrier.

17. A method for treating a patient having an IGF associated condition comprising administering to the patient the biologically active recombinant IGF-I produced in accordance with claim 1.

18. The method of claim 17, wherein the biologically active recombinant IGF-I is in an acceptable pharmaceutical carrier.

19. A method for treating a patient having an IGF associated condition comprising administering to the patient the biologically active recombinant IGF-I produced in accordance with claim 11.

20. The method of claim 19, wherein the biologically active recombinant IGF-I is in an acceptable pharmaceutical carrier.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/02457

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K3/08; C12N15/18; A61K37/36; C12P21/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; A61K ; C12P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 327 503 (CIBA-GEIGY AG) 9 August 1989 see claims 1-28 ---	16-19
Y	EP,A,0 364 926 (BOEHRINGER MANNHEIM GMBH) 25 April 1990 see page 4, line 1 - line 20 ---	1-15
Y	EP,A,0 359 163 (THE GENERAL HOSPITAL CORPORATION) 21 March 1990 see page 12, line 55 - page 13, line 40; claims 1-11; tables 2,3 ---	13-15
Y	EP,A,0 219 874 (BOEHRINGER MANNHEIM GMBH) 29 April 1987 see page 5, left column, line 24 - page 15, right column, line 56 ---	1-15

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 JULY 1993	25 -08- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HORNIG H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	EP,A,0 135 094 (AMGEN) 27 March 1985 see page 23, line 18 - page 24, line 14	1-15
Y	EP,A,0 130 166 (KABIGEN AB) 2 January 1985 cited in the application see page 16, line 1 - page 17, line 2	1-15
A	PROTEIN PURIFICATION: MICRO TO MACRO 1987, ALAN LISS, INC.,NEW YORK, US; R. BURGESS & Proceedings of a Cetus-UCLA Symposium, Frisco, Colorado, March 29-April 4, 1987; M.W. KNUTH and R.R. BURGESS: " Purification of proteins in the denatured state" see page 292, line 37 - page 293, line 35	1-12

INTERNATIONAL SEARCH REPORT

Interr nal application No.
PCT/US 93/02457

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos..
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 17-20 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/ composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos..
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9302457
SA 71701

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 09/07/93

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